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Developmental changes in melanophores and their asymmetrical responsiveness to melanin-concentrating hormone during metamorphosis in barfin flounder (*Verasper moseri*)

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Research Highlights

(1) MCH response in vitro was assessed in barfin flounder during metamorphosis.

(2) MCH responsiveness was first confirmed in larval-type melanophores in teleosts.

(3) On ocular side, MCH target cells changed from larval- to adult-type melanophores.

(4) On blind side, larval-type melanophores retained MCH responsiveness in juveniles.

(5) Inhibited development was proposed as the key mechanism for blind side formation.

Abstract: Barfin flounder larvae exhibit unique black coloration, as well as left-right asymmetry in juvenile stage as in other flatfish. In this study, we first assessed the changes in melanophores with development and then investigated their responsiveness to melanin-concentrating hormone (MCH) during metamorphosis. Larval-type melanophores appeared on both sides of the body before metamorphosis, whereas adult-type melanophores appeared only on the ocular side after metamorphosis. Even in the individuals of this species displaying black coloration, the density of larval-type melanophores was similar to that in transparent larvae of other species. However, unlike in transparent larvae, larval-type melanophores completely dispersed in the black larvae of this species. Therefore, the black coloration during larval stages was mainly due to dispersion, and not the density, of larval-type melanophores. In vitro MCH treatment revealed, for the first time, the responsiveness of melanophores in larval stages. On the ocular side, larval-type melanophores aggregated against MCH during larval stages, while, in the larvae at later metamorphic stages and in juveniles, larval-type melanophores did not aggregate, although aggregation of adult-type melanophores was noted. In contrast, on the blind side, the responsiveness of larval-type melanophores to MCH was consistently present from larval to juvenile stages. The metamorphic transition of MCH responsiveness from larval- to adult-type melanophores only on the ocular side suggests the larval (therefore, immature) nature of the blind side skin. We propose that the inhibited development, and thus the retention of the larval-type skin leads to the formation of the blind side characteristics and is the central mechanism for the flatfish asymmetry.

Keywords: Melanin-concentrating hormone (MCH); Flatfish; Metamorphosis; *Verasper moseri*; Melanophore; Asymmetrical responsiveness

1. Introduction

Flatfish are known to display cryptic changes in coloration on the ocular side. These changes are caused by the rapid aggregation or dispersion of pigments within the chromatophores, which is controlled by the neuroendocrine or sympathetic nervous system (Fujii and Oshima, 1986; Fujii, 2000). Flatfish represent a phylogenetically derived order of Teleostei; their exceptional ability to change color is an important evolutionary adaptation among the diversity of teleost coloration (Burton, 2010). Flatfish exhibit left-right asymmetry in coloration during metamorphosis from symmetrical larvae to asymmetrical juveniles. The evolutional origin of flatfish is a symmetrical ancestor (Norman, 1934). Thus, their ability to asymmetrically change coloration might have been acquired during the course of evolution.

Barfin flounder, *Verasper moseri*, is a large flatfish inhabiting the cold sea basin around northeastern Japan. During development, the larvae at stages A–F (from yolk sac to flexion larvae) are in the pelagic phase, and the eye migration begins at stage G. During stages H–I, they settle to the bottom (Aritaki et al., 2000). Hence, for settled larvae and juveniles, changing their coloration to match the ground color is reasonable. However, our preliminary observation revealed that they actually cease to change coloration for a few weeks. Furthermore, most larvae at stage G exhibit black coloration as described previously (Aritaki et al., 2000), whereas some were transparent in our observation. These developmental changes suggest that some physiological changes occur in the neuroendocrine and sympathetic nervous systems during metamorphosis.

Elucidation of these changes requires the basic information on pigmentation development in barfin flounder. Barfin flounder larvae exhibit black coloration, although larvae of most other fish are transparent. Therefore, investigating the pigmentation mechanisms in barfin flounder is interesting since it might provide some insights into larval coloration and their pigment-controlling systems. Previous studies on flatfish chromatophores only focused on species that have transparent larvae, such as Japanese flounder (Seikai et al., 1987), stone flounder (Matsumoto and Seikai, 1992), and summer flounder (Bolker and Hill, 2000). Body coloration develops on the basis of the number,

status, and distribution of 3 types of chromatophores: melanophore, xanthophore, and iridophore (Bolker and Hill, 2000). In addition, melanophores are classified into 2 types: the large larval-type and the small adult-type (Seikai et al., 1987). Larval-type melanophores are present on both the sides during the larval stage, whereas adult-type melanophores appear only on the ocular side after metamorphosis.

In neuroendocrine systems, aggregation or dispersion of pigments is controlled by 2 different peptides having opposite activities, melanin-concentrating hormone (MCH) and melanocyte-stimulating hormone (MSH; Burton, 2002; Fujii, 2000). MCH, a 17-amino acid peptide, was first identified in fish and is known to regulate changes in coloration (Kawauchi et al., 1983). In various teleosts, including barfin flounder, MCH aggregates melanophores in adults or juveniles (Kawauchi, 2006; Mizusawa et al., 2011; Takahashi et al., 2004; Yamanome et al., 2007). However, information on larval responsiveness to MCH is very limited except for the study on rainbow trout (Suzuki et al., 1997). In barfin flounder, MCH neuronal somata and fibers were first detected in the pituitary 7 days after hatching (DAH, stage B; Amano et al., 2003). Therefore, MCH possibly aggregates larval-type melanophores in this species. In contrast, although MSH is also known to disperse melanin granules (Fujii, 2000; Fujii and Oshima, 1986), their activity might not be detected under the possible predominance of MCH and the sympathetic nervous system in flatfish (Burton, 2002; Mizusawa et al., 2011).

In the present study, we determined the changes in melanophores during development and investigated their responsiveness to MCH during metamorphosis. Our results showed that changes in MCH responsiveness of larval-type melanophores occurred only on the ocular side, suggesting that the blind side skin retained larval (therefore, immature) characteristics. This finding is discussed in relation to the formation of the blind side.

2. Materials and methods

2.1 Fish

106

107 Artificially fertilized eggs of barfin flounder (*V. moseri*) were obtained from Date Station of
108 Hokkaido Aquaculture Promotion Corporation. The eggs were incubated in a 100-L polycarbonate
109 tank at 8°C. After hatching, larvae were stocked in 1000-L polycarbonate tanks, initially at 8°C and
110 gradually the temperature was increased to 14°C at a rate of 1°C/day; they were fed rotifers 10–25
111 DAH and *Artemia* nauplii 26–55 DAH. Side and bottom walls of the tanks were transparent, but
112 covered with a white heat-insulating plate. Rearing tanks were set indoor under natural photoperiod
113 of the rearing location (about 15L: 9D). The classification of the developmental stages was as
114 suggested by Aritaki et al. (2000). Larvae (stages E–I) and juveniles (75 DAH) were sampled for
115 each 5–10 fish. However, since the number of available larvae of definitive stage H was not enough,
116 due to the relatively short duration of the stage and available timing of the experiment, and since
117 morphological difference between stages H and I is small, these 2 stages were pooled in some
118 experiments.

119

120 *2.2 Melanophore density and status*

121

122 Chromatophores were photographed without anesthetizing the fish by using a dissecting light
123 microscope (M216; Leica, Germany) equipped with a digital camera (DFC300FX; Leica).
124 Melanophores and xanthophores were distinguished by the black and yellow coloration.
125 Melanophores were classified into 2 groups: large larval-type melanophores and small adult-type
126 melanophores (Seikai et al., 1987). The number of larval- and adult-type melanophores in five
127 1-mm^2 areas near the base of the caudal fin was counted for each individual. The degree of pigment
128 aggregation or dispersion in melanophores was evaluated using melanophore index (MI; Hogben and
129 Slome, 1931). In brief, MI = 1.0 corresponded to the most aggregated status; and MI = 5.0, to the
130 most dispersed state. About 30 or more melanophores of the larval type, without or with
131 considerably more number of adult–type melanophores were focused in each photograph obtained
132 using our microscope system; therefore, 30 melanophores per individual were examined for

133 determining the MI for both the types of melanophores. Since melanophores at stage G rapidly
134 dispersed by handling stress, the MI was evaluated when the larvae turned black and had completed
135 the color change.
136
137 *2.3 In vitro responsiveness to MCH*
138
139 Barfin flounder MCH (Takahashi et al., 2004) was synthesized and purchased from GL
140 Biochem (Shanghai) Ltd. (Shanghai, China). Fish were immobilized in ice-cold seawater, and then
141 the posterior half of the body (tail) was severed by using a sharp razor blade as described by Suzuki
142 et al. (1997). Immediately after the tail was removed, the fish were killed by cutting off their heads.
143 The tails were washed in balanced salt solution (BSS; Burton and Vokey, 2000) containing HEPES
144 (pH 7.5), and then incubated in 1 mL of BSS containing MCH (0–1000 nM) for 30 min at 20°C in a
145 well of a 24-well culture plate. Aggregation or dispersion of melanophores was investigated as
146 described above.
147
148 *2.4 Statistical analysis*
149
150 The values are shown as means ± standard error. Melanophore density and MI were analyzed
151 using Mann–Whitney *U* test to compare the differences between ocular and blind sides. The variance
152 in the MI was analyzed using Kruskal–Wallis test by using XLSTAT 2012
153 (http://www.mindware-jp.com/xlstat/). When significant differences were detected, post hoc
154 Steel–Dwass test was applied to compare among the groups ($p < 0.05$). Non-parametric tests were
155 used for MI, because it is based on a ranking scale.
156
157 **3. Results**
158
159 *3.1 Melanophore density on the ocular and blind sides during development*

Larval-type melanophores were found at a density of 80 cells/mm^2 on both sides at stage E, and the density decreased consistently with development to a level of 25 cells/mm^2 (Fig. 1A). At each stage, no significant differences were observed between the ocular and blind sides. In contrast, adult-type melanophores appeared only on the ocular side from stage I at a level of 300 cells/mm^2 (Fig. 1B).

3.2 Melanophore aggregation or dispersion status during development and its response to handling

During development, melanophore aggregation or dispersion status markedly changed. When the larvae were black (stages E–G), larval-type melanophores completely dispersed (MI = 5.0) on both the sides (Fig. 2A and B). However, when the black color became pale and expressed patterns on the ocular side (stages H–I and juveniles), larval-type melanophore aggregation was noted (MI = 3.0–4.0; Fig. 2A and B). Only at stage G, color variation among individuals was observed. About 70% of larvae exhibited black coloration similar to that noted during stages E–F, but about 30% were transparent.

In the transparent larvae of stage G, aggregation of larval-type melanophores was noted (MI = 1.0; Fig. 3A). After handling, the transparent larvae dramatically changed coloration to black within 45 s, due to the dispersion of the larval-type melanophores (Figs. 3A–C, MI = 1.0 and 5.0 before and after the handling, respectively). However, at stage I, no such drastic changes in coloration were observed after handling (Fig. 3D–F, MI = about 3–4, both before and after the handling). Similarly, no changes in coloration were observed in the larvae of stages E and F (data not shown).

3.3 In vitro responsiveness to MCH during development

The experimental system for larval responses to MCH was validated by investigating time- and dose-dependent responses by using the tails of stage F larvae (Figs. 4 and 5). Since blood

187 circulation was absent and melanophores were not exposed on the skin surface, delivery of MCH
188 from the incubation medium to melanophores required diffusion. The time lag between the addition
189 of MCH and arrival of MCH to melanophores was reduced by using a high dose of MCH (1,000
190 nM). The color began to change from black to pale within 5 min (Fig. 4B), and the entire area of the
191 tail became pale after 30 min (Fig. 4C). No further changes were observed after 90 min (Fig. 4D). In
192 the dose-dependent response experiment, tails of stage F larvae were incubated in MCH (0–1,000
193 nM) for 30 min. No significant aggregations were observed in the control larvae and larvae treated
194 with 1 nM MCH. However, larvae treated with 10–1,000 nM MCH showed significant aggregation
195 of larval-type melanophores (Fig. 5).
196 Next, the tails of larvae from each stage were incubated in MCH (1,000 nM) for 30 min. For G
197 stage, black larvae were used. Development-related changes in response of larval-type melanophores
198 to MCH, as well as left-right asymmetry, were noted in larvae of stages H–I and juveniles (Fig. 6).
199 On the ocular side, larval-type melanophores aggregated significantly only at stages E–G, but not in
200 larvae at stages H–I and juveniles (Fig. 6A). In contrast, on the blind slide, significant aggregation of
201 larval-type melanophores was noted at all stages (Fig. 6B). On the other hand, aggregation of
202 adult-type melanophores was noted in stage I larvae and juveniles, although their MI values were not
203 estimated because the magnification of the photographs was extremely low (Fig. 7).
204
205 **4. Discussion**
206
207 *4.1. Coloration control during larval and juvenile stages in barfin flounder*
208
209 Barfin flounder larvae exhibit unique pigmentation development within the diversity of teleost
210 coloration. Interestingly, the larvae exhibit black as well as asymmetrical coloration. Our results
211 provide strong evidence that the aggregation or dispersion of larval-type melanophores plays a major
212 role in this unique coloration. Thus far, pigmentation development in fish has been intensively
213 studied using zebrafish (Quigley and Parichy, 2002); flatfish may serve as a model because their

214 pigmentation undergoes a repatterning at metamorphosis.

215 The black coloration is represented by quantitative differences in the density or status of
216 melanophores. In barfin flounder, both melanophores and xanthophores appeared on the skin (data
217 not shown), similar to the findings of a previous study conducted on the transparent larval flatfish
218 (Bolker and Hill, 2000). In addition, transparent larvae of both Japanese flounder (Nakamura et al.,
219 2010) and summer flounder (Bolker et al., 2005) exhibited almost identical density of larval-type
220 melanophores as those found in black larvae of barfin flounder (Fig. 1A). However, at stages E–G,
221 dispersion and aggregation status of larval-type melanophores was significantly different between
222 the 2 species: completely dispersed melanophores (MI = 5.0) in barfin flounder (Fig. 2), whereas MI
223 = 2.0–3.0 (Yoshikawa, unpublished) in Japanese flounder. Therefore, the black coloration in dark
224 larvae might be due to the dispersion of larval-type melanophores and not their density. Although
225 xanthophores might also contribute to the dark larval coloration in barfin flounder, their density and
226 status were not investigated due to the interference by the extensive dispersion of larval-type
227 melanophores in this study.

228 Our results suggested that MCH significantly aggregated larval-type melanophores. To our
229 best knowledge, this is the first report showing MCH responsiveness of larval-type melanophores in
230 the fish that have both larval- and adult-type melanophores. Early responsiveness (as early as 3
231 DAH) of melanophores to MCH has been shown in rainbow trout (Suzuki et al., 1997); however, the
232 presence of 2 types of melanophores has not been confirmed in salmonids. Larval-type
233 melanophores significantly aggregated in the presence of as low as 10 nM MCH (Fig. 5). Such
234 responses are consistent with a study on peppered catfish and Nile tilapia, which showed
235 melanophore aggregation at 10 nM MCH as the lowest concentration (Kawauchi and Baker, 2004).
236 Although larval-type melanophores remained dispersed at stages E–F (Fig. 2) in nature, they are
237 expected to have MCH receptors at those stages, because they aggregated by MCH treatment.

238 Our results suggested that the black coloration could be related to the MCH concentration in
239 the circulation. In barfin flounder, MCH neuronal somata and fibers were first detected in the
240 hypothalamus and pituitary, respectively, at 7 DAH (stage B; Amano et al., 2003). The early

projection of MCH fibers might indicate active secretion of MCH into the circulation. However, in normal development, larval-type melanophores first aggregated at stage G (Fig. 3A). Further, the distribution of MCH neuronal somata and fibers first displayed a pattern similar to that in the adult fish 35–42 DAH (stages G–H; Amano et al., 2003), which could be the beginning of active secretion of MCH. Thus, MCH in the circulation might be low at stages E–F. In addition, in cichlid fish, MCH immunoreactivity was detected in the neuromast of larval heads, and the possibility of paracrine control of melanophores was suggested (Pandolfi et al., 2003). For the larval coloration and its possible control, MCH level in circulation and possible production of MCH in peripheral tissues, as well as the interactions of MSH or sympathetic nervous systems, needs to be investigated.

The responsiveness of larval-type melanophores completely disappeared from the ocular side at the latter half of metamorphosis, irrespective of the influence of the neuroendocrine or sympathetic nervous system. Our in vitro investigation of MCH responsiveness revealed that larval-type melanophores aggregated only at stages E–G, and no aggregations were observed at stages H–I and in juveniles (Fig. 6A). This result indicated that the responsiveness of larval-type melanophores to MCH on the ocular side disappears at stages H–I. Under the control of the sympathetic nervous system, larval-type melanophores did not disperse after handling stress at stages H–I (Fig. 3D and E), whereas they showed remarkable dispersion in transparent larvae of stage G (Fig. 3A–C). Since this dispersion was considerably faster than that noted after exposure to neuroendocrine hormones such as MCH (Fig. 4), the lack of rapid dispersion after handling stress probably indicates the lack of responsiveness to sympathetic nervous system activity. After the responsiveness of larval-type melanophores on the ocular side disappeared, adult-type melanophores appeared only on the ocular side (Fig. 1B) and exhibited MCH responsiveness (Fig. 7). Internal tissues such as skeletal muscles and erythrocytes are known to undergo morphological changes from larval- to adult-type during metamorphosis (Inui et al., 1995). Similarly, our results might indicate the transition of MCH responsiveness on the ocular side from larval- to adult-type during metamorphosis.

268 *4.2. Larval (immature) nature of the blind side skin in flatfishes*

269

270 The left-right asymmetry of flatfish has attracted considerable interest; however, the central
271 mechanisms underlying the metamorphic asymmetry have not been elucidated. During melanophore
272 development in symmetrical fish such as zebrafish, the second wave of melanophore differentiation
273 occurs at the time of metamorphosis (Johnson et al., 1995). This is similar to the differentiation that
274 occurs on the ocular side of flatfish, where adult-type melanophores differentiate during
275 metamorphosis following larval-type melanophores (Fig. 1; Seikai et al., 1987). In other words, the
276 blind-side skin has a unique pattern of development. Although the thyroid hormone is known to
277 generally stimulate flatfish metamorphosis and create an asymmetrical body (Inui et al., 1995), the
278 timing of thyroid hormone action determines the success or failure of the blind side formation
279 (Tagawa and Aritaki, 2005), again suggesting the uniqueness of the blind side development. Taken
280 together, these findings suggest that flatfish have acquired a unique mechanism to create the blind
281 side characteristics in the course of evolution.

282 The asymmetrical response to MCH was similar to the developmental transition pattern of
283 other asymmetrical tissues and organs of flatfish. As described above, MCH responsiveness changed
284 from larval- to adult-type on the ocular side (Figs. 6 and 7), but was unchanged and remained
285 larval-type on the blind side even after metamorphosis (Fig. 6). There are several other examples
286 similar to MCH responsiveness: differentiation of adult-type melanophores (Seikai et al., 1987),
287 formation of ctenoid scales (Kikuchi and Makino, 1990), increase of mucus cells (Seikai, 1992),
288 increase of epidermal filament-containing cells (Suzuki, 1994), degeneration of intraepithelial blood
289 vessels (Suzuki, 1994), and degeneration of chloride cells (Suzuki, 1994). All these adult
290 characteristics of the ocular side appear after metamorphosis, and larval (immature) characteristics
291 remain on the blind side. This corroborates the idea presented by Suzuki (1994): “The asymmetrical
292 formation process is thought to be due the cell differentiation on the obverse side and
293 pseudomorphism on the reverse side through the adaptive characteristics in relation to a bottom life
294 style.”

295 Even in normally metamorphosed juveniles of flatfish, some portion of the blind side skin
296 sometimes becomes dark, especially in those fish that are reared in tanks without bottom sand. This
297 phenomenon is called “staining” (Norman, 1934). Since the stained area has ocular side
298 characteristics for scale types and pigment cell populations (Isojima et al., 2013), it is clear that the
299 blind side skin of larval-type has a potential to develop into ocular side skin of adult-type even after
300 metamorphosis. Therefore, the ocular side skin (having adult-type skin commonly found in various
301 teleosts) might be considered to be in the terminal phase, whereas the blind side skin (having
302 larval-type skin) is in the transient phase. Thus, a possible inhibition mechanism that stops the
303 development of adult-type characteristics occurring on either side (future blind side) of the body is
304 strongly suggested as the central mechanism for the formation of flatfish asymmetry.
305
306 **5. Conclusions**
307
308 Our study showed the population and status changes in melanophores during development and
309 their responsiveness to MCH. Melanophore dispersion was found to be the possible reason for the
310 black coloration found in some teleost larvae. In addition, this is the first report suggesting the
311 presence of responsiveness to MCH in definitive larval-type melanophores, along with the
312 disappearance of this responsiveness only on the ocular side. The metamorphic transition of MCH
313 responsiveness from larval- to adult-type melanophores only on the ocular side suggests the larval
314 (immature) nature of the blind side skin. We propose that the inhibited development, and thus the
315 retention of larval-type skin leads to the formation of the blind side characteristics and is the central
316 mechanism for the establishment of flatfish asymmetry.
317
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319
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329

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409

410 **Figure legends**

411

412 Fig. 1. Developmental changes in melanophore density. Larval-type melanophores (A) and
413 adult-type melanophores (B). Black and white circles show the ocular and blind sides,
414 respectively. The values are shown as means of 5 fish ± standard error. The asterisk shows
415 statistical significance between ocular and blind sides; $p < 0.05$, Mann–Whitney U test.
416 Inserted photographs show larval-type (A, blind side of stage I individual) and adult-type (B,
417 ocular side of stage I individual) melanophores, respectively. Scale bars indicate 50 μm.

418 Fig. 2. Developmental changes in the aggregation/dispersion of larval-type melanophores. Ocular
419 side (A) and blind side (B). The values are shown as means of 5 fish ± standard error.
420 Different letters show statistical significance; $p < 0.05$, Kruskal–Wallis test with a post hoc
421 Steel–Dwass test.

422 Fig. 3. Changes of body coloration and larval-type melanophores after handling stress. The larva at
423 stages G and I, before (A, D), 45 s (B, E), and 180 s (C, F) after the handling stress. Black
424 arrowheads indicate larval-type melanophores. Scale bars indicate 5 mm (upper) and 500 μm
425 (lower).

426 Fig. 4. Time course response to MCH (1,000 nM). The coloration of larval tails of stage F before (A)
427 and 5 min (B), 30 min (C), and 90 min after (D) incubation. Scale bar indicates 1 mm.

428 Fig. 5. Dose response to MCH in larval-type melanophores at stage F. Black and white bars show
429 MCH and control groups, respectively. The values are shown as means of 3–5 fish ± standard
430 error. Letters show statistical significance; $p < 0.05$, Kruskal–Wallis test with a post hoc
431 Steel–Dwass test.

432 Fig. 6. Developmental changes in larval-type melanophore response to MCH (1,000 nM). Ocular
433 side (A) and blind side (B). Black and white bars show MCH and control groups, respectively.
434 The values are shown as means of 5 fish ± standard error. Asterisks show statistical

435 significance between control and MCH groups; $p < 0.05$, Mann–Whitney *U* test.

436 Fig. 7. Adult-type melanophore response to MCH (1,000 nM) at stages H–I. Small black arrows and

437 large black arrowheads indicate adult- and larval-type melanophores, respectively. Scale bar, 1

438 mm.

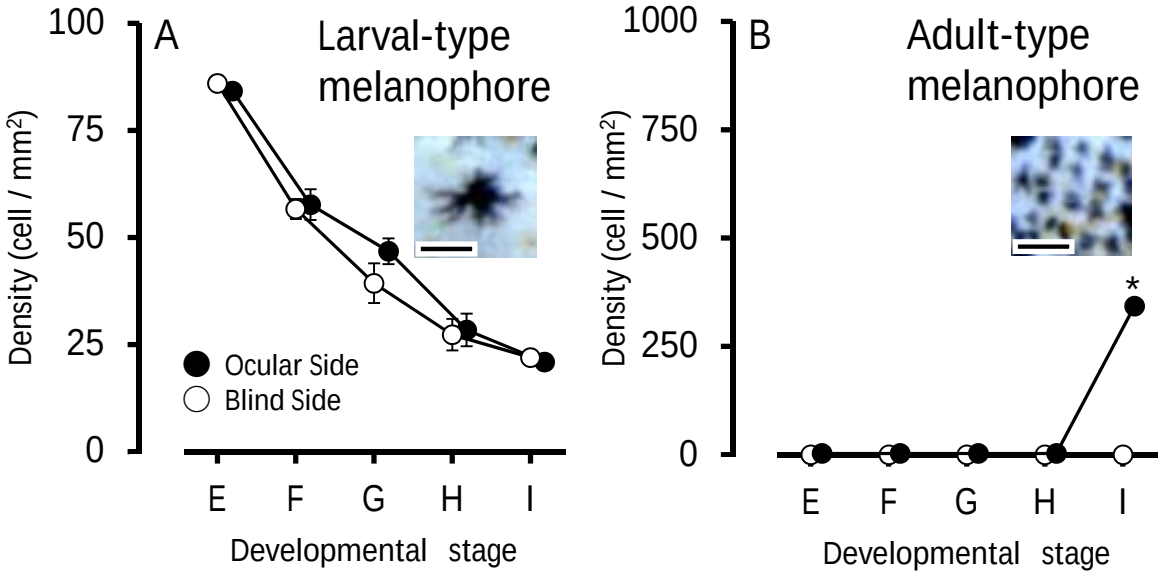
A
Larval-type
melanophore
100
75
50
25
0
Density (cell / mm2)
Ocular Side
Blind Side
E
F
G
H
I
Developmental stage
B
Adult-type
melanophore
1000
750
500
250
0
Density (cell / mm2)
*
E
F
G
H
I
Developmental stage

Fig.1

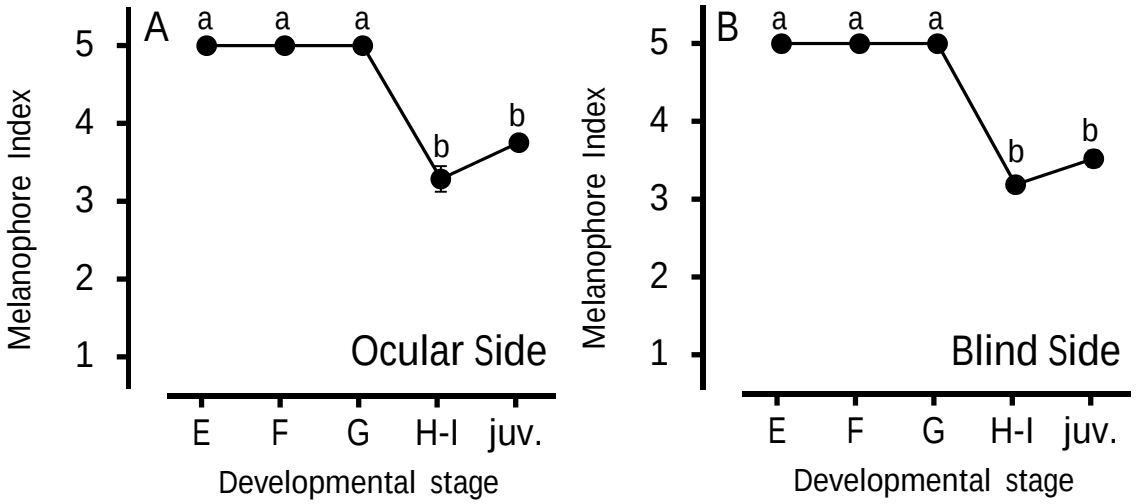
A
a
a
a
b
b
5
4
3
2
1
Melanophore Index
Ocular Side
E
F
G
H-I
juv.
Developmental stage
B
a
a
a
b
b
5
4
3
2
1
Melanophore Index
Blind Side
E
F
G
H-I
juv.
Developmental stage

Fig.2

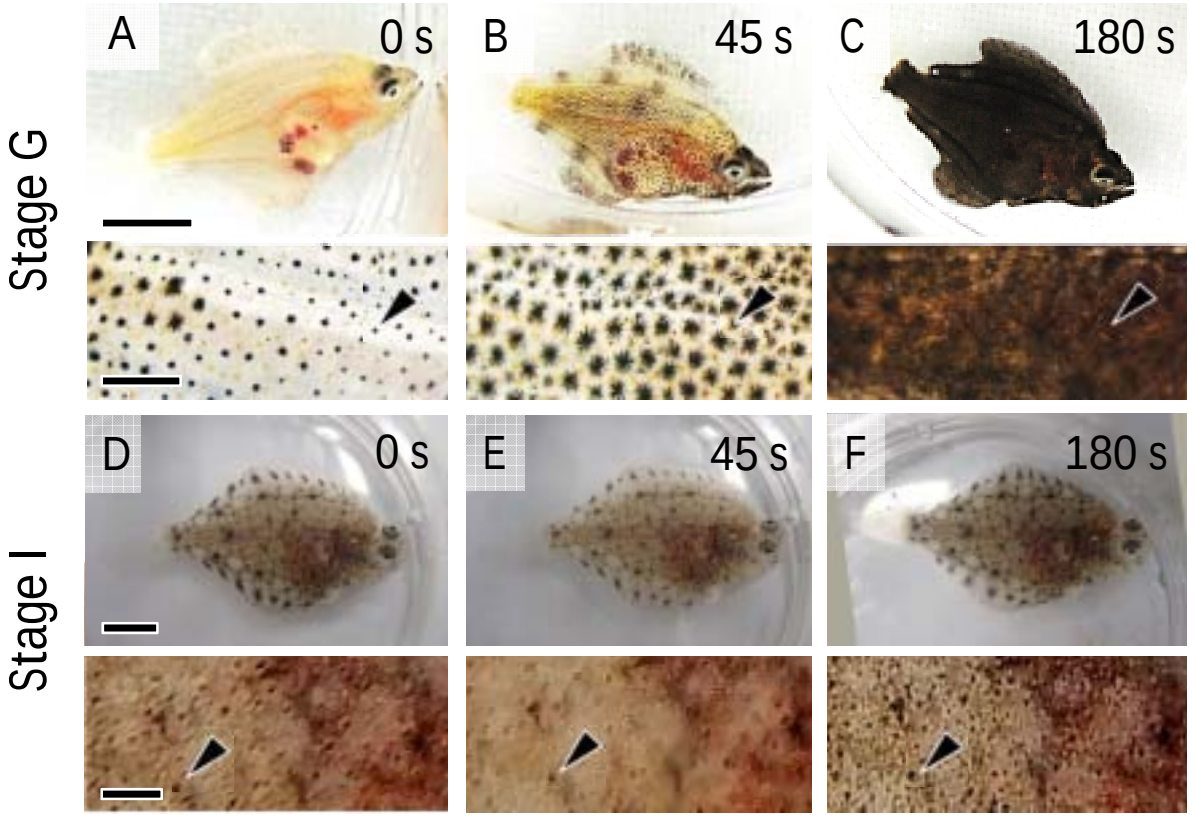

Fig.3

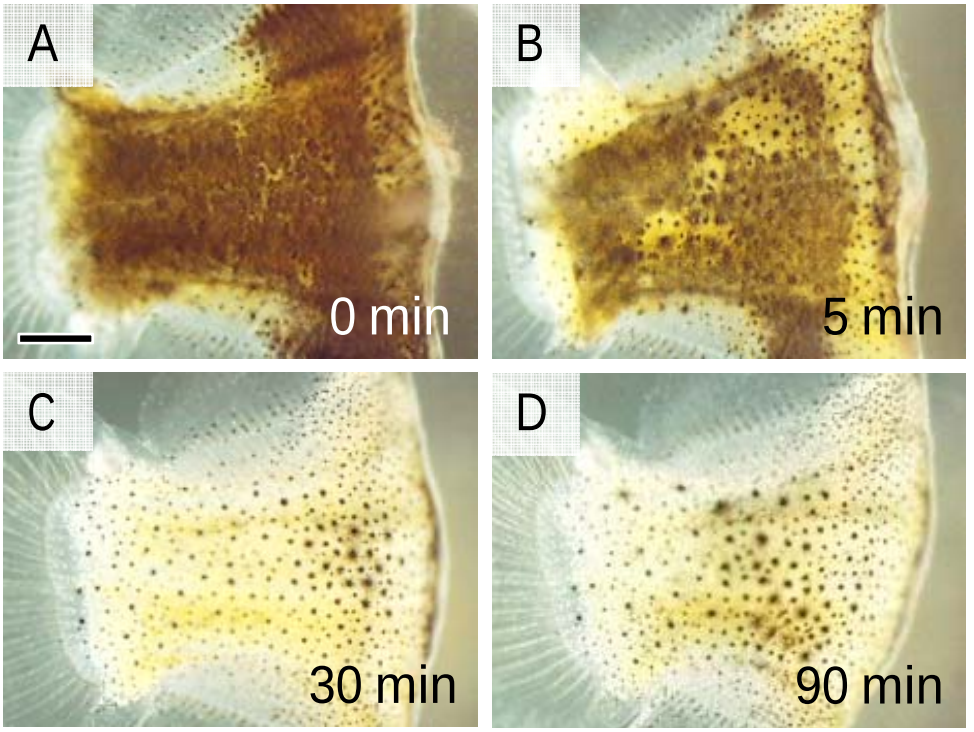

Fig.4

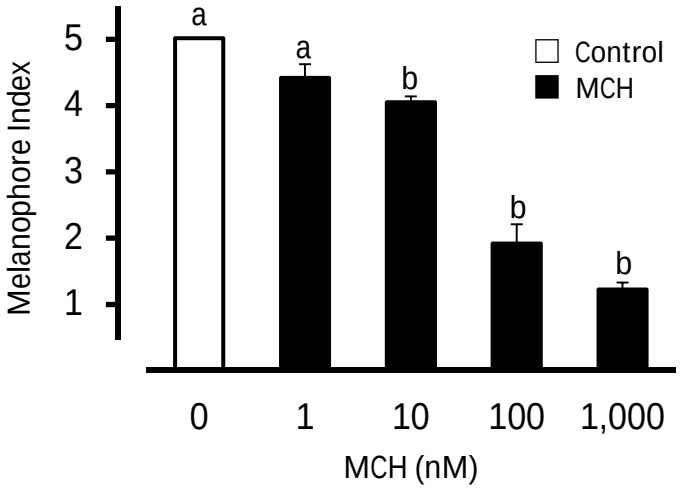
Melanophore Index
5
4
3
2
1
a
a
b
b
b
Control
MCH
0
1
10
100
1,000
MCH (nM)

Fig.5

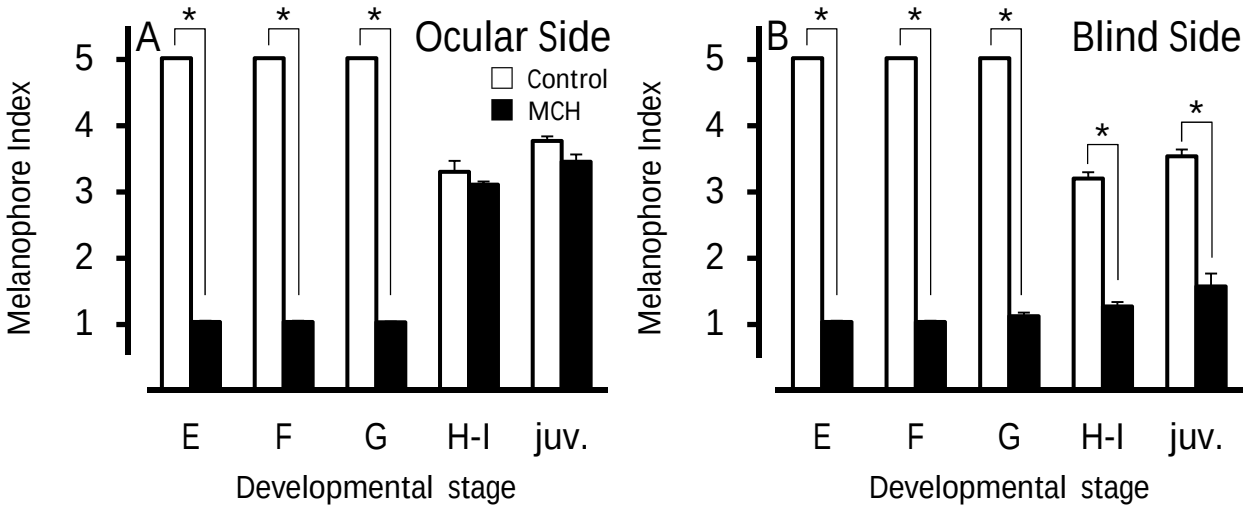
A
Ocular Side
Control
MCH
Melanophore Index
5
4
3
2
1
*
*
*
E
F
G
H-I
juv.
Developmental stage
B
Blind Side
Melanophore Index
5
4
3
2
1
*
*
*
*
*
E
F
G
H-I
juv.
Developmental stage

Fig.6

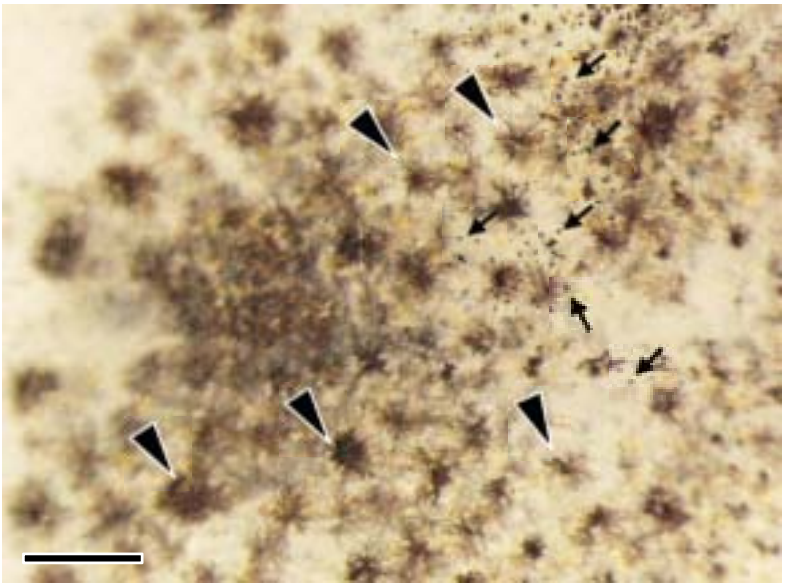

Fig.7